Title: A method for identifying a *Mycobacterium* species

The invention relates to a method for identifying a *Mycobacterium* species responsible for a mycobacterial infection in a human or animal, and to diagnostic kits for use in said method.

5   The genus *Mycobacterium* contains about 50 species. It is responsible for a number of diseases which are known collectively as mycobacterioses. The best known and widest spread of these are leprosy, caused by *M. leprae*, and tuberculosis caused by *M. tuberculosis*. Both of these

10   diseases affect more than ten million people all over the world. Most other mycobacteria normally occur only as environmental saprophytes. However, they can also cause opportunist diseases, which happens often, but not exclusively, in organisms suffering from problems with their

15   immune systems, such as AIDS patients or people undergoing immunosuppression. The opportunist types comprise the slow-growing species *M. avium*, and the closely related *M. intracellulare* and *M. scrofulaceum* (often together referred to as the MAIS complex), *M. kansai*, *M. marinum* and *M.*

20   *ulcerans*, and the fast-growing species *M. chelonae* and *M. fortuitum*. Although quite rare in the Western world for several decades, the occurrence of opportunist mycobacterial diseases and tuberculosis has shown a significant increase with the incidence of AIDS. Further, it has been reported

25   that mycobacteria or antigens of mycobacteria play a role in the etiology of a plurality of other diseases, such as sarcoidosis and Crohn's disease, as well as different auto-immune diseases, such as auto-immune dermatitis, rheumatoid arthritis and diabetes. It has been suggested that this role

30   can be attributed to a structural mimicry between epitopes of mycobacteria and those of the host organism.

The cell walls of mycobacteria are very complex and contain many different lipids, some of which have structures unique to the genus. These structures comprise mycolinic

acids and esters, peptido-glycolipide, arabino-galactane and lipo-arabinomanane. The lipid-rich cell walls of a mycobacterial cell are responsible for the notable coloring properites of the mycobacteria. They also enable mycobacteria to counter an attack by the immune system of a host organism. A number of species, after being taken up into macrophages, surround themselves with a thick layer of secreted lipids.

Many of the different components of the mycobacteria interact with the immune system of a host organism. These components comprise proteins and hydrocarbon antigens, which can either be actively secreted by the mycobacteria or can form part of the cell wall or cell membrane. In addition, they may be present in the cytoplasm, for example in the cytoplasmic matrix, ribosomes and enzymes. Mycobacteria further also possess immuno-modulating components, such as immunosuppressing compounds and adjuvants. As of consequence, a single mycobacterial species can induce a large variety of immune responses in different forms having diverse specificities. This makes it very difficult to derive protein antigens which are suitable for the detection of species-specific humoral responses as a basis for a highly sensitive and specific diagnostic test for the above mentioned diseases, particularly tuberculosis. Because mycobacteria have a frequent occurrence, both human and animal body fluids contain nearly at all times anti-mycobacterial antibodies.

In the past, researchers have attempted to develop a sufficiently sensitive diagnostic test for mycobacterioses. The focus of these attempts has mostly been on finding species-specific glycolipid antigens for the detection of specific humoral immune responses, because of the problems with the specificity of protein antigens.

In the international patent application 94/14069, it has been disclosed to make use of the antibody response of an organism to immuno-dominant mycobacterial cross-reactive antigen components (further referred to as Im-CRAC) for developing a diagnostic test for mycobacterial infections.

The Im-CRAC is believed to provide indirect information on the nature of the immune recognition of, and response to, a specific mycobacterial pathogen.

5   The method proposed in WO-A-94/14069 is based on the discovery that the clinical manifestation of mycobacterial diseases is related to the varying capability of an individual host to produce a humoral response to different mycobacterial immuno-cross-reactive antigen components (Im-CRAC). Each mycobacterial infection generates its own
10   specific antibody response to a number of specified antigens. Analysis of the antibody-response, e.g. by immunoblotting, has demonstrated that the immuno-dominant Im-CRAC vary in accordance with the immunopathological manifestation of the mycobacterial diseases. Said analysis results in different
15   and distinguishing band patterns of mycobacterial antigens for different individuals which are infected with different *Mycobacterium* species. The band pattern, which is obtained after an immunoblot, can be referred to as an Antigen Bar Code. The antigen-antibody reactions which are shown in the
20   bar code are, when taken together, unique for a certain *Mycobacterium* species.

The present invention aims at providing an improved method for identifying a *Mycobacterium* species in a diagnostic test. Although satisfactory in most aspects, it is
25   still desirable to have a diagnostic test which is even more sensitive than the method described in WO-A-94/14069. Furthermore, there is a need for a diagnostic test which can be used in the determination of previous vaccination, and for monitoring therapy of organisms infected with a *Mycobacterium*
30   species. During therapy, there are situations where low levels of certain antibodies occur, which may disturb the accuracy and/or sensitivity of the test making use of the antibody-antigen cross-reactions, as outlined hereinabove.

It has now been found that a highly sensitive
35   diagnostic test can be performed by contacting a sample of a body fluid with both antibodies and antigens. It has been

found that, besides antibodies, several mycobacterial components are present in animal and human body fluids, of which the presence can be determined by using cross-reactions with a chosen set of antibodies in a reliable manner.

5   The invention therefore provides a method for identifying a *Mycobacterium* species comprising the steps of:

a) contacting at least one immuno-cross reactive antigen component of a mycobacterial species with a sample of a body fluid of a human or animal individual;

10   b) contacting at least one antibody, which is capable of reacting with a mycobacterial antigen, with said body fluid sample;

c) detecting the presence of antigen-antibody complexes, and identifying the *Mycobacterium* species present

15   in said body fluid sample.

Surprisingly, a highly reliable test has been developed based on the concept as outlined above. By using the method of the invention it is possible to monitor the different stages of a treatment of a mycobacterial disease.

20   Also, it is possible to determine whether an individual has been vaccinated for a mycobacterial disease, and for which mycobacterial disease. As not every organism infected with a *Mycobacterium* species shows the same reaction to said infection, a very reliable test is provided, in that

25   a wide area of both antigens and antibodies that may be present can be covered in the test method.

The present method can be applied to any sample of body fluid of any human or animal individual. Of course, the most reliable results are obtained when a sample of a body

30   fluid wherein the presence of a *Mycobacterium* species is most often encountered. Examples of suitable body fluids include serum, blood and excretion fluids, such as sputum, saliva, CSF (cerebrospinal fluid), or tear fluid. These body fluids can either be subjected to a method according to the

35   invention directly, or they can undergo some form of pretreatment. Usually, the body fluids will be diluted by

dissolution in a buffer solution prior to the diagnostic test.

In a preferred embodiment, saliva or a solution or preparation thereof is subjected to a diagnostic test for identifying a *Mycobacterium* species. If necessary, the mucus-like structure of the saliva can be removed by a treatment with for instance xylomethasolin or by any other known method. The invention also encompasses a diagnostic test method for identifying a *Mycobacterium* species wherein a sample of saliva is contacted with at least one immuno-cross-reactive antigen component of a mycobacterial species, and/or at least one antibody which is capable of reacting with a mycobacterial antigen, detecting the presence of antigen-antibody complexes, and identifying the *Mycobacterium* species present in the saliva sample. Surprisingly, the high content of various enzymes in saliva does not disturb the reliability of the diagnostic test to any substantial extent. In the alternative, the effect of the enzymes on the outcome of the test can suitably be annulled.

The performance of a test on saliva has surprisingly proven to be very successful and reliable. Apparently, mycobacterial infections are sufficiently manifested in saliva. The collection and use of saliva has many advantages over the use of more conventional body fluids, such as serum or blood. It is relatively easy to collect, even under the difficult field conditions, that are often encountered in Third World countries where both tuberculosis and leprosy have high incidence. Furthermore, saliva is non-invasive, which raises compliance of individuals to be tested, requires minimal training to collect and has reduced biohazard risk in collection, transport and testing, in particular in areas where a high incidence of HIV-infections exists.

As has been set forth hereinabove, according to the present invention a sample of a body fluid is contacted with both an antigen or a preparation thereof and an antibody or a preparation thereof. Very good results have also been

obtained with a test wherein a sample of a body fluid is contacted with only an antibody, which is capable of reacting with a mycobacterial antigen, or a preparation thereof. The inventions thus also encompasses a method for identifying a

5   *Mycobacterium* species comprising the steps of contacting at least one antibody, which is capable of reacting with a mycobacterial antigen, detecting the presence of antibody-antigen complexes, which have been formed between said antibody and one or more antigen components of the

10   *Mycobacterium* species, and identifying the *Mycobacterium* species present in said body fluid sample.

In accordance with the invention, it is possible to contact a body fluid sample first with the antigen and subsequently with the antibody or vice versa. It is

15   preferred, however, that the sample of a body fluid is contacted with the antigen and the antibody simultaneously. This can, for instance, be done by passing the body fluid sample by two surfaces, to one of which the antigen or the preparation thereof has been bound, and to the other of which

20   the antibody or the preparation thereof has been bound. On the basis of this principle, the skilled person will be able to develop various ways of performing the diagnostic test. It will be apparent that when the method is chosen such that the antigen or the preparation thereof and the antibody or the

25   preparation thereof are in contact with one another, the antigens and antibodies used have to be chosen such that they do not react with each other.

The at least one immuno-cross-reactive antigen component may comprise the total or a fraction of the

30   components of *Mycobacterium* or the total or a fraction of the culture medium of *Mycobacterium*. In principle, any fraction of *Mycobacterium* or a culture medium thereof can be used. Such a fraction can be obtained in a conventional manner, and is advantageously separated by electrophoresis prior to the

35   method according to the invention as has been described in WO-A-94/14069 to provide a banding pattern after the test has

been performed. Usually, the antigen component will mainly comprise lipo-polysaccharides. However, often proteins will also be present. According to the invention, both lipo-polysaccharides and proteins, having antigen action, can be
5 used separately or combined.

Preferred antigen components are chosen from the non-exhaustive group of KATG, MPT63 (=18 kD), MPT64 (=24 kD), MPT51, MTC28, Ag85a (=30-31 kD), Ag85b (=Ag6), Ag85c, Ag5 (=CIE Ag78 and =38kD), DES, MPB70,80 (=22/23 kD),
10 Lipoologosaccharide (LOS), lipoarabinomannan (LAM), PMB67 (67 kD), Isocitrated dehydrogenase, Malate dehydrogynase, 2,3-diacyl-trehalose (DAT), Phenolicglycolipid (PGL), ESAT6 (=6 kD), hsp70 = DnaK = Ag63 (=71kD), CIE Ag82 = GroEL and homologues (=65 kD), GroES and homologues = BCGa (=10kD),
15 Antigen60, as well as those having molecular weights of 6 kD, 10/12 kD, 16 kD, (often referred to as 14 kD), 18 kD, 19 kD, 21 kD, 22 kD, 23 kD, 24 kD, 28 kD, 29 kD, 30 kD, 30 kD, region 32 kD, 33 kD, 34 kD, 36 kD, 38 kD, 42 kD, 50-55 kD, 60 kD, 65 kD, 67 kD, 71 kD, 88 kD, and 95 kD.
20 Preferably, the at least one immuno-cross-reactive antigen component comprises a KP90, KS90, antigen6, KP100, or SP100 fraction, or a fraction of a culture medium of a preparation of a Mycobacterium species. It has been found that these antigen preparation provide very reliable test
25 results. Moreover, they are sufficiently stable to be stored for a prolonged period of time without affecting the reliability of the diagnostic test.

The at least one antibody for a mycobacterial species with which the sample of a body fluid is contacted in
30 accordance with the invention, can be any antibody which is capable of reacting with a certain mycobacterial antigen. Such an antibody or a preparation thereof can be obtained in a manner known to the skilled artisan. According to the invention, it is preferred to use monoclonal antibodies,
35 although the use of polyclonal antibodies has also proven to be suitable. Preferably, IgG, IgA, IgM or a combination

thereof is used as the antibody. These antibodies have proven
to be very convenient to handle in a diagnostic test and lead
to very satisfying results.

In a preferred embodiment, the at least one immuno-
5   cross-reactive antigen component or the at least one antibody
for a mycobacterial species is immobilized on a support. Both
non-solid supports and solid supports can be used.
Preferably, a solid support is used.

Particularly preferred is the embodiment wherein both
10   the antigen component and the antibody are immobilized on a
support. When steps a) and b) are performed simultaneously, a
particularly useful test has proven to be based on an
embodiment of the invention wherein the antigen component and
the antibody are immobilized on the same support. As has been
15   stated above, in that case it is necessary that the antibody
does not react with the at least one immuno-cross-reactive
antigen component. It is advantageous to provide a layer of
the at least one immuno-cross-reactive antigen component on
top of a layer of the at least one antibody, or vice versa.
20   Preferably, first a layer of the at least one immuno-cross-
reactive antigen component is applied to the solid support,
and then a layer of the at least one antibody is applied
thereon.

The at least one immuno-cross-reactive antigen
25   component will generally be used in a concentration from 0.1
to 20 µg/ml, and preferably from 1 to 10 µg/ml. When the
antigen component is applied to a support, the amount of said
component will depend on the test and the support chosen. The
at least one antibody will generally be used in a
30   concentration from 0.1 to 20 µg/ml, and preferably be from 1
to 10 µg/ml. When the antibody component is applied to a
support, the amount of said component will depend on the test
and the support chosen. The ratio wherein the at least one
immuno-cross-reactive antigen component and the at least one
35   antibody are used, will usually be of from 1:10 to 10:1,
preferably from 1:2 to 2:1.

Preferred solid supports are chosen from the non-exhaustive group consisting of membranes, such as nitrocellulose membranes, dip-sticks, filters, spheres, granules and microtiter plates. The antigen component and or
5   the antibody may be immobilized on these supports in a manner known in the art.

After the sample of the body fluid has been contacted with the at least one immuno-cross reactive antigen component and the at least one antibody, there may or may not have
10   formed antibody-antigen complexes. These complexes can be of two types; either the antibody or the antigen component of these complexes will be from the body fluid. Both or just one type of these complexes may be present. The detecting of the two types of complexes can be carried out separately or
15   together. Preferably, they are performed simultaneously. Suitable detecting methods are immunoblotting, as has been disclosed in WO-A-94/14069, as well as any of the usual direct and indirect labeling methods known in the art. Suitable labels can be chosen from the non-exhaustive group
20   of biotin, biocytin, iminobiotin, digoxigenin, avidin, streptavidin, colloidal dye substances, fluorochromes, such as rhodamin, reducing substances, such as eosin or erythrosin, (colored) latex sols, carbon sols, metals, metal sols or other particulate sols, dansyl lysin, Infra Red Dyes,
25   coumarines, enzymes, and iodide labels. Particularly preferred is the use of gold labels, colloid labels, latex sols, and enzymes. The use of these labels enable the performance of a method according to the invention in a so-called rapid test.
30   After the analysis, the Mycobacterium species can suitably be identified. Although any conventional identification procedure can be used, it is preferred that the Mycobacterium species is identified on the basis of one or more reference patterns.
35   Of course, the invention also encompasses a diagnostic kit for use in a method as described hereinabove.

The kit comprises a support, on which at least one immuno-cross reactive antigen component of a mycobacterial species and at least one antibody, which is capable of reacting with a mycobacterial antigen and which does not react with said at least one immuno-cross reactive antigen component, are bound, and means for detecting the presence of antigen-antibody complexes. The support is preferably chosen from the group of membranes, dip-sticks, filters, spheres, granules and microtiter plates. The means for detecting may comprise the means for detecting in a manner as described herein above.

The invention will now be elucidated by the following non-restrictive examples.

EXAMPLES

Preparation of KP90/KS90

KP90 was prepared from starting material from crude mycobacterial mass as described in WO-A-94/14069 (the contents of which are incorporated herein by reference). Starting material made from 1 gram lyophilized bacteria was centrifuged at 90,000 x g at 4°C for 2 hours. The pellet was washed two times with PBS. Between the washing steps the sample was sonicated for 6x30 seconds on ice with interruptions of 10 seconds and centrifuged at 90,000 x g at 4°C for 2 hours. The pellet was collected and resuspended in 10 ml 0.05 M Tris buffered saline (TBS) pH 7.4. The supernatants, designated as KS90, can also be used as antigen. The pellet was sonicated for 6x15 seconds on ice with interruptions of 10 seconds. This preparation is designated with the term KP90. After the protein concentration was determined, quantities of 1.7 ml were frozen at concentrations of 1 mg/ml in 25 mM TBS/50% glycerol and stored at -20°C. The following components have been shown to be present in KP90: LAM (++), 10 kD (+), 16 kD (+), 21 kD (+), 24 kD (?), 30 kD (+/-), 31 kD (-), 34 kD (+/-), 38 kD (-), 65 kD (++), and 95 kD (+).

Preparation of a culture fluid fraction

After culturing *Mycobacterium tuberculosis* for 3 weeks, the bacteria were removed by centrifugation and filtration.

5 Components from the culture fluid were precipitated by 0-45% ammonium sulphate precipitation. After centrifugation, the precipitate was dialyzed and further purified by ion exchange chromatography. This fraction is called antigen6. Further purification can be performed by hydrophobic interaction

10 chromatography.

Preparation of monoclonal antibodies (MoAb)

IgG monoclonal antibodies were prepared according to the literature (Clin. Exp. Immunol. (1984) 58:511-521)

15

Preparation of saliva

Saliva was collected using the Omni-Sal™ saliva collection device (Saliva Diagnostic Systems) and stored at -20°C. The tube contains 1 ml of a preservative solution. The pad is

20 designed to hold 1 ml of fluid when saturated, resulting in a 1:2 dilution of saliva. Before use the saliva sample was pretreated with protease inhibitor and triton X and nonidet P40 (0.01%)

Preparation of serum

25 Blood was obtained by venipuncture and processed to serum using standard methods.

EIA

30 **Coat**

Before coating KP90 was sonicated for 5x10 seconds on ice with 10 seconds interruptions.
Microtiter plates were coated with KP90 in several dilutions in PBS pH8.0 for 22 hrs at 37°C, or with one or more MoAbs

35 (IgG or IgM) against components of *M. tuberculosis* in several dilutions in PBS pH 8.0 overnight at 4°C. MoAbs that were

tested were a.o.: a MoAb against a 38kD protein of *M. tuberculosis*, and a MoAb against LAM. Combination coatings of MoAbs and KP90 were made by coating first with KP90 and then with one or more MoAbs, or by first coating with KP90 on one half of the microtiter plate well, while the plate was placed under an angle followed by coating with one or more MoAbs after the plate was turned 180°.

After coating the plates were blocked with 3% BSA for 1 hour at room temperature dried at 37°C and stored at 4°C.

Test

Sera were tested in a 1:200 and 1:400 dilution. Saliva samples were tested in several dilutions ranging from 1:1 to 1:100.

100 µl was pipetted into the coated wells of a microtiter plate and incubated for 1 hour at 37°C. Non-binding serum components are washed away in a washing step with PBS. A second incubation with a conjugate, either anti-Hu-IgA, or anti-Hu-IgM, labeled with peroxidase, or another MoAb against the same component of *M. tuberculosis* which is monitored labeled with peroxidase, or a combination of these two is performed for 1 hour at 37°C. Excess conjugate is then washed away.

Indication of the presence of human antibodies of the sub-type IgA or IgM binding specifically to KP90 and/or the presence of *M. tuberculosis* components in the sample takes place by adding TMB (tetramethylbenzidine) to the wells.

Binding enzyme results in the occurrence of a blue color which, after addition of a coloring stop solution, changes to yellow. This yellow color has an absorption maximum of 450 nm.

Interpretation of the test results takes place on the basis of the so-called cut-off sample. A test sample can be considered positive when the result found in the test scores higher than the cut-off sample. The cut-off sample is based

on results with a large panel of positive and negative subjects.

Results

5   After performing the procedures described above under 'Test' and 'Coat', the results as shown in Table I were obtained regarding the presence of antibodies in saliva. For determining anti-KP90-IgA, a saliva dilution of 1:100 was find to be suitable. For determining anti-KP90-IgG, a saliva

10   dilution of 1:20 appeared to be suitable. In Table I, samples 38, 39, 40, 44, 4,18, 25, 31, and 33 were TB-positive, whereas the other samples were TB-negative.

       A combination of an anti-*Mycobacterium* IgA and IgG antibody detection is saliva, wherein different cut-offs are

15   employed, has additional value in diagnostics. Also, testing saliva in combination with serum has been found to have additional value in *Mycobacterium* diagnostics.

Table I: detection of anti-*Mycobacterium* IgA and IgG in saliva samples

Saliva	IgA (saliva 1:100) OD450	+/- bij OD CO=0,7	IgG (saliva 1:20) OD 450	+/- bij OD CO=0,22
1	0.689	-	0.074	-
2	0.345	-	0.064	-
3	0.302	-	0.050	-
5	0.321	-	0.065	-
6	0.462	-	0.063	-
7	0.141	-	0.071	-
9	0.509	-	0.075	-
10	0.609	-	0.066	-
12	0.446	-	0.162	-
15	0.173	-	0.054	-
16	0.335	-	0.108	-
22	0.561	-	0.100	-
23	0.389	-	0.096	-
28	0.177	-	0.064	-
32	0.305	-	0.178	-
34	0.683	-	0.173	-
35	0.55	-	0.213	-
36	0.567	-	0.070	-
38	0.742	+	0.234	+
39	0.351	-	0.364	+
40	0.555	-	0.301	+
44	0.801	+	0.235	+
4	1.074	+	1.152	+
18	0.833	+	0.106	-
25	1.064	+	0.678	+
31	0.059	-	2.204	+
33	1.945	+.	0.077	-

In order to determine the presence of LAM in saliva, saliva was first prepared as described above. Additionally, 1 ml samples were dialyzed in order to remove triton and salt, whereafter they were freeze-dried and taken up in 200 µl PBS. Of the thus obtained samples, 100 µl was pipetted in each well of a microtiter plate provided with a coat as described above. Detection took place as described above using rabbit anti-*M. tuberculosis* antibodies, which were in

their turn detected using anti-rabbit-IgG-peroxidase conjugate. The results are shown in Table II.

Table II: detection of LAM in saliva

Saliva		OD1	OD2	mean
1	TB-positive	0.924	0.97	0.947
2	TB-positive	0.737	0.79	0.764
3	TB-positive	0.472	0.453	0.463
4	TB-positive	0.722	0.734	0.728
5	TB-positive	0.439	0.431	0.435
6	TB-positive	0.599	0.603	0.601
7	TB-positive	0.72	0.693	0.707
8	TB-positive	0.69	0.565	0.628
9	TB-positive	0.643	0.669	0.656
mean				0.659
10	TB-negative	0.289	0.285	0.287
11	TB-negative	0.353	0.338	0.346
12	TB-negative	0.294	0.293	0.294
13	TB-negative	0.296	0.294	0.295
14	TB-negative	0.298	0.297	0.298
15	TB-negative	0.315	0.309	0.312
16	TB-negative	0.35	0.34	0.345
17	TB-negative	0.353	0.344	0.349
18	TB-negative	0.253	0.261	0.257
mean				0.309

Further, IgG, IgA, and LAM were detected in sputum samples analogous to what has been described above for saliva. Anti-*M. tuberculosis* antibodies were determined in sputum using KP90.

To detect antibodies in sputum, samples of sputum were diluted 1:20 or 1:25 (for IgG and IgM) or 1:100 (for IgA) in serum dilution buffer from the KREATECH IgA EIA kit. The samples were vigorously shaken for at least one hour. Subsequently, the samples were tested for the presence of anti-tuberculosis antibodies by ELISA as described above.

KP90 was coated on a microtiter plate. The cut off ratio was 0.8 for IgG and IgM and 0.6 for IgA with serum 8-59 as a reference to calculate the ratio. The results are shown in

Table III.

Table III: detection of antibodies in sputum

Sputum		IgG	IgA	IgM
1	TB-negative	negative	positive	negative
2	TB-negative	negative	positive	negative
3	TB-negative	negative	negative	negative
4	TB-negative	negative	negative	negative
5	TB-negative	negative	positive	negative
6	TB-negative	negative	negative	negative
7	TB-negative	negative	negative	negative
8	TB-negative	negative	negative	negative
9	TB-positive	positive	positive	positive
10	TB-positive	positive	negative	positive
11	TB-positive	positive	negative	negative
12	TB-positive	negative	positive	positive

5

Western blot

Gelelectrophoresis and preparation of membrane for assays was performed as described in WO-A-94/14069, page 13 under point 2.

10   Strips were incubated with 1:100 or 1:200 diluted human serum or saliva collected with an Omni-Sal device dilutions ranging from 1:1 to 1:100.

For the detection of antibodies in serum and saliva immunodetection was performed as described in WO-A-94/14069.

15

Dot Blot Assay

Antigen (KP90) 1-10 μg/ml and MoAb 0.5-5 μg/ml were spotted separately onto nitrocellulose membrane. The membrane

20   (blocked or unblocked with BSA) was incubated with serum (1:200 dilution) or saliva sample (1:1-1:100 dilution) in PBS/tween/BSA for 1 hour at room temperature. After washing the membrane with PBS/tween the membrane was incubated incubation with conjugate:

25   1. indirect label:

a combination of anti-Hu-IgA or anti-Hu-IgG labeled with peroxidase and a MoAb (IgG or IgM) against the same component of *M. tuberculosis* which is monitored labeled with peroxidase. Detection was performed using AEC substrate (0.8%
5   3-amino-9-ethylcarbazole in dimethylformamide) 1:10 diluted in AEC buffer (50mM Acetate Buffer, pH5.0, 0.1% ureumperoxide), DAB (3,3'-diamino-benzidine tetrahydrochloride) using standard methods, or the ECL detection system of Amersham.
10 2. direct label:
a combination of anti-human-IgA or anti-human-IgG labeled with gold and a MoAb (IgG) against the same component of *M. tuberculosis*, which is monitored, labeled with gold. The IgA-gold conjugate was obtained commercially or made as described
15 in e.g. WO-A-96/35696. The MoAb-gold conjugate was made as follows: The gold particles were obtained commercially. The conjugate was made according to standard procedures or in the case of platinum-based linker as described in e.g. WO-A-96/35696.
20

Rapid Strip test

Antigen (KP90) 1-10 μg/ml and one or more MoAbs (IgG) 0.5-5 μg/ml are immobilized on different lanes on nitrocellulose membrane. A control lane depending on the Ab
25 which is detected (anti-IgA or anti-IgG/proteinA) is also applied above the other lines. The nitrocellulose is used blocked with 0.1% BSA or unblocked. On a attached absorbent pad anti-IgA-gold conjugate and MoAb-gold conjugate (Moab against the same antigen as the immobilized MoAb but
30 recognizing another determinant) are dried. The IgA-gold conjugate was obtained commercially or made as described in WO-A-96/35696. The MoAb-gold conjugate was made as described above. The sample (serum or saliva) is diluted in 1 ml PBS (pH 7.4) in an glass tube. The test strip is then dropped
35 into the tube with the absorbent pad downwards. After 15 min to 2 hours, when the control line was colored, the results

were determined.

Second procedure: Antigen (KP90) 1-10 µg/ml and one or more MoAbs (IgG) 0.5-5 µg/ml are immobilized on different lanes on nitrocellulose membrane (see figure 1). A control
5   lane depending on the Ab which is detected (anti-IgA or anti-IgG/proteinA) is also applied above the other lines. An absorbent pad was attached. The whole sample was labeled with gold conjugate through a platinum based linker (see e.g. WO-A-96/35696). Then the strip was dropped into the sample
10   with the absorbent pad downwards. After 15 min to 2 hours, when the control line was colored, the results were determined.

15   **Agglutination test**
coating

The latex particles are washed several times with Borate (pH 8.5) or PBS, by filtration, or by use of ion-exchange resins depending on the quantity needed. Antigen (KP90 or
20   antigen6) or antibody are coated on latex particles by incubating, for 1-16 hours at 37°C, 20°C or 4°C, 1 ml of 1% suspension of the appropriate particles in Borate (pH 8.5) or PBS containing 0.01% tween-20 and a final protein concentration up to 5 mg/ml. Subsequently the latex is
25   centrifuged and the supernatant containing any unabsorbed ligand is discarded. After several washing steps (see above) the coated latex is re-suspended in MES buffer containing 0.1% BSA and can be used in a latex agglutination test or stored at +4°C.
30

Agglutination

The coated latex particles are mixed in several dilutions with serum or saliva sample in a drop on a glass slide. Agglutination is assayed after 1 at room temperature to 16
35   hours at +4°C incubation time. The agglutination can be scored by eye (floc appearance) or after filtration of the

mixture. In the latter case a pore size of the membrane is chosen which allows non agglutinated latex particles to pass and retains agglutinated particles. By using colored particles the membrane is colored in case of a positive
5   reaction.

By using different colored latex particle for antigen (KP90 or antigen6) and antibody both reactions can be monitored separately.

10   **Results**

Serum was tested in accordance with the above procedure using KP90 and polystyrene particles. The incubation time of the test was from 5 minutes at room temperature. The results are shown in Figure 1. Figure 1A shows a positive reaction,
15   whereas Figure 1B shows a negative reaction.